Title

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A *trans*10-18:1 enriched fraction from beef fed a barley grain-based diet induces lipogenic gene expression and reduces viability of HepG2 cells

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ABSTRACT

Beef fat is a natural source of *trans (t)* fatty acids, and is typically enriched with either *t*10-18:1 or *t*11-18:1. Little is known about the bioactivity of individual *t*-18:1 isomers, and the present study compared the effects of *t*9-18:1, *cis* (*c*)9-18:1 and *trans (t)*-18:1 fractions isolated from beef fat enriched with either *t*10-18:1 (HT10) or *t*11-18:1 (HT11). All 18:1 isomers resulted in reduced human liver (HepG2) cell viability relative to control. Both *c*9-18:1 and HT11were the least toxic, *t*9-18:1had dose response increased toxicity, and HT10 had the greatest toxicity ($P < 0.05$). Incorporation of *t*18:1 isomers was 1.8–2.5 fold greater in triacylglycerol (TG) than phospholipids (PL), whereas Δ9 desaturation products were selectively incorporated into PL. Culturing HepG2 cells with *t*9-18:1 and HT10 increased ($P < 0.05$) the Δ9 desaturation index (*c*9–16:1/16:0) compared to other fatty acid treatments. HT10 and *t*9-18:1 also increased expression of lipogenic genes (FAS, SCD1, HMGCR and SREBP2) compared to control ($P < 0.05$), whereas *c*9-18:1 and HT11 did not affect the expression of these genes. Our results suggest effects of HT11 and *c*9-18:1 were similar to BSA control, whereas HT10 and *t*-9 18:1 (i.e. the predominant *trans* fatty acid isomer found in partially hydrogenated vegetable oils) were more cytotoxic and led to greater expression of lipogenic genes.

1. Introduction

Consumption of *trans* (*t*) fatty acids have been associated with numerous adverse effects such as increased plasma LDL-cholesterol/HDL-cholesterol ratio, inflammation, insulin resistance, endothelial dysfunction and oxidative stress [1,2]. The main dietary source of *trans* fatty acids has been partially hydrogenated vegetable oil (PHVO), which can contain up to 60% *trans* fatty acids. The *trans* fatty acids in PHVO are mainly in the form of *trans*-octadecenoic acid (*t*-18:1), representing 90–95% of total *trans* fatty acids in PHVO [3]. Among these, *t*9-18:1 (elaidic acid) and *t*10-18:1 are typically the first and second major isomers, making up on average about 28% and 21% of total *t*-18:1 respectively [4]. Internationally, reduction in PHVO in the food supply has become a priority, and this is to the point where PHVO are no longer generally recognized as safe in the USA, and food manufactures have been given three years to take them out of their products [5]. This leaves ruminant meat and milk as the major source of *t*18:1 in human diets.

In ruminant animals (e.g. sheep, cattle, goats), *t*18:1 isomers are produced by rumen microbes during biohydrogenation of dietary polyunsaturated fatty acids (PUFA), and these can be incorporated into meat and milk [6]. When cattle are fed diets with a high forage to grain ratio, *t*11-18:1 is typically 50–70% of *t*-18:1 isomers, but when a low forage to grain diet is fed, ruminal biohydrogenation pathways shift towards *t*10-18:1 production [7]. The most concentrated *trans* fatty acid in ruminant products is,

Abbreviations: ACC, acetyl-CoA carboxylase; Ag+-SPE, silver ion solid phase extraction; BSA, bovine serum albumin; *c*,, *cis*; FAS, fatty acid synthase; HMGCR, 3-Hydroxy-3-Methylglutaryl-CoA reductase; HT10, high-*t*10 fraction; HT11, high-*t*11 fraction; MUFA, monounsaturated fatty acids; PHVO, partially hydrogenated vegetable oils; PL, phospholipid; PUFA, polyunsaturated fatty acids; SCD1, stearoyl-CoA desaturase-1; SFA, saturated fatty acid; SREBP1c, sterol regulatory element-binding protein-1c; SREBP2, sterol regulatory element-binding protein-2; *t*, *trans*; TG, triacylglycerol; TLC, thin layer chromatography

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however, frequently assumed to be *t*11-18:1 (vaccenic acid), and this has made it difficult to interpret their human health effects. In contrast to PHVO, epidemiological studies indicate ruminant *trans* fatty acids are not associated with increased risk for coronary heart disease [8–11], and several animal and cell culture studies have noted positive health effects of *t*11-18:1. Clinical trials, however, have shown *trans* fatty acids from both PHVO and ruminant fats have adverse effects on blood lipids and lipoproteins [12,13].

Studying the effects of *t*18:1 isomers in cell culture is made difficult because some isomers (e.g. t9- and *t*11-18:1) are commercially available, while others (e.g. *t*10-18:1) are not. In order to clarify effects of ruminant products with differing *trans* fatty acid composition, a limited number of animal feeding studies have included dairy products enriched with different *t*-18:1 isomers [14,15]. Changes in the *t*-18:1 profile, however, may have led to confounding effects due to changes in the saturated fatty acid (SFA) and *cis*-monounsaturated fatty acid (*c*-MUFA) contents. To alleviate confounding effects of other fatty acids, we developed silver-ion chromatography techniques to isolate *t*-18:1 fractions and individual *t*-18:1 isomers in quantities sufficient to test their metabolism and bioactivity in cell culture [16]. The objective of the present experiment was to compare the effects of *t*18:1 fractions from beef fat enriched with *t*10-18:1 (HT10) or *t*11-18:1 (HT11) with *t*9-18:1 (the major *t*18:1 isomer in PHVO) and *c*9-18:1 (oleic acid) in liver cell culture (HepG2). We chose liver cells because of their central role in the metabolism of *cis* and *trans* fatty acids including β-oxidation, Δ9 desaturation and lipoprotein secretion [17]. In addition, the adverse effects of *trans* fatty acids from PHVO on blood lipoproteins have been suggested to be mediated in part via modulation of hepatic lipogenic gene expression [18,19]. We hypothesized that beef *t*-18:1 fractions with different *t*-18:1 isomer profiles would have distinct effects on HepG2 cells, specifically in terms of cell viability, lipogenic gene expression, and incorporation of *t*-18:1 isomers into cell triacylglycerol (TG) and phospholipid (PL) fractions.

2. Materials and methods

2.1. Fatty acid treatments

The HT11 and HT10 fractions were isolated from banked backfat samples collected from cattle fed forage (hay) and barley grain-based diets respectively. Diets included supplements rich in 18:2n-6 to increase the content of *t*18:1 fatty acids in beef fat. The distribution of *t*-18:1 isomers of the HT11 and HT10 fractions are presented in Table 1. *Trans*-18:1 fractions were isolated using silver ion (Ag^+) solid phase extraction [16]. Briefly, fat was freeze dried, dissolved in toluene and methylated using 0.5 M sodium methoxide [20]. The resulting fatty acid methyl esters were dissolved in hexane and applied to Discovery® Ag^+-SPE columns (750 mg/6 ml, Supelco, Bellefonte, PA, USA). Saturated fatty acids (SFA) were eluted with 10 ml of hexane and the *t*-18:1 fraction was collected with 10 ml 98:2 hexane: acetone (v/v). The t-18:1 FAME were then saponified to free fatty acids using 0.3 M methanolic potassium hydroxide [21].

Table 1
Composition of *t*-18:1 fractions used for HeG2 culture.

Fatty acid (%)	HT10[a]	HT11[a]
*t*6–*t*8-18:1	7.4	4.0
*t*9-18:1	5.7	4.1
*t*10-18:1	69.3	2.9
*t*11-18:1	7.6	65.4
*t*12-18:1	1.8	6.9
*t*13/*t*14-18:1	3.0	6.8
*t*15-18:1	0.7	5.1
*t*16-18:1	0.4	2.9

[a] HT11 = *t*-18:1 fraction enriched with t11-18:1 from a beef cattle fed a grass-hay based diet. HT10 = *t*-18:1 fraction enriched with t10-18:1 from a beef cattle fed a barely grain based diet.

Individual 18:1 isomers including *t*9-18:1 and *c*9-18:1 were purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA). Individual fatty acids and *t*18:1 fractions were complexed with fatty acid free bovine serum albumin (BSA) at a 4:1 M ratio (4 mM fatty acid: 1 mM BSA) as described by Evans et al. [22]. Fatty acid-BSA complexes were then diluted in cell culture media to provide desired final fatty acid concentrations (100 or 200 μM).

2.2. Cell viability

To test viability, cells were cultured with growth medium containing 5% FBS and supplemented with 100 μM or 200 μM of fatty acids for 96 h. Cell proliferation was assessed using CellTiter-Blue cell viability assay (Promega, Madison, WI). Briefly, HepG2 cells were seeded in 96-well plates (~1000 cells/well) and allowed to attach before 300 μl of medium containing 100 or 200 μM of fatty acid treatment was added. At 0 h, 24 h, 48 h, 72 h and 96 h growth medium including fatty acid treatments was replaced with 100 μl/well serum free medium plus 20 μl/well CellTiter 96® AQueous One Solution reagent (Promega, Madison, WI). After 1.5 h incubation at 37 °C in 6% CO2, the absorbance at 490 nm was recorded using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). The viability results for fatty acid treatments were expressed relative to BSA control.

2.3. Culture conditions for gene expression and fatty acid analyses

Human hepatoma HepG2 cells (ATCC; Rockville, MD, USA) were seeded at a density of 1×10^5 cells per well (9.6 cm^2) in 6-well plates, and cultured at 37 °C in 6% CO2 in a growth medium containing Eagle's minimum essential medium (EMEM; ATCC) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich, St. Louis, MO, USA) and 1% penicillin-streptomycin (Life Technologies, Burlington, ON, Canada). Cell culture medium was changed every two days, and at 24 h post-confluence, FBS was removed from the medium and cells were treated with 100 μM of the fatty acid-BSA complex for 24 h, and cells were used for fatty acid and gene expression analyses. Control cells were cultured with an equal volume of BSA (vehicle control). Two wells of cells were cultured per treatment per experiment, and the experiment was repeated three times.

2.4. RNA extraction, reverse transcription and quantitative PCR

Total RNA was extracted from HepG2 cells using the Aurum total RNA fatty and fibrous tissue kit (Bio-Rad Laboratories, Mississauga, ON, Canada). The RNA concentrations were determined by absorbance at 260 nm using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), and the RNA purity was evaluated using the 260:280 and 260:230 absorbance ratios to ensure both ratios were between 1.8 and 2.1. Integrity of RNA was confirmed by the presence of intact RNA subunits 28S and 18S using an automated capillary electrophoresis QIAxcel system (Qiagen, Hilden, Germany). cDNA was synthesized from 1 μg of RNA using M-MLV reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) in the presence of random hexamer primers and Ribonuclease Inhibitor (Invitrogen) in a total reaction volume of 20 μl. Real-time PCR analysis was performed using a Stratagene Mx3005P QPCR system (Agilent Technologies) using

Table 2
Gene specific forward and reverse primer sequences of genes used for Real-Time PCR.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
ACC	GAGGGCTAGGTCTTTCTGGAAG	CCACAGTGAAATCTCGTTGAGA
FAS	TATGCTTCTTCGTGCAGCAGTT	GCTGCCACACGCTCCTCTAG
SCD1	CTCCACTGCTGGACATGAGA	AATGAGTGAAGGGGCACAAC
HMGCR	TACCATGTCAGGGGTACGTC	CAAGCCTAGAGACATAAT
SREBP1c	GCGGAGCCATGGATTGCAC	CTCTTCCTTGATACCAGGCCC
SREBP2	CGCCACCTGCCCCTCTCCTTCC	TGCCCTGCCACCTATCCTCTCACG
β-actin	AAAGACCTGTACGCCAACACAGTGCTGTCTGG	CGTCATACTCCTGCTTGCTGATCCACATCTGC

ACC: acetyl-CoA carboxylase; FAS: fatty acid synthase; SCD1: stearoyl-CoA desaturase-1; HMGCR: 3-Hydroxy-3-Methylglutaryl-CoA reductase, SREBP1c: sterol regulatory element-binding protein-1c; SREBP2: sterol regulatory element-binding protein-2.

the following protocol: enzyme activation 95 °C for 10 min, initial denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 60 s, repeated for 40 cycles. This was followed by a melt curve analysis as per the manufacturer's settings to ensure specific amplification. Primers were designed using primer 3.0 software or taken from the literature (Table 2).

Samples were run in triplicate in 96-well plates, and each 20 µl reaction contained 6 µl cDNA diluted at 1:50, 0.4 µl of each forward and reverse primes (10 mM), 8 µl Green-2-Go qPCR Mastermix (BioBasic, Markham, ON, Canada), and 5.2 µl of nuclease-free water. Relative mRNA expression was calculated using ΔCt method with β-actin as internal control gene. Briefly, target gene cycle threshold (Ct) values was normalized to that of β-actin using $2^{-\Delta Ct}$ where ΔCt=Ct target gene−Ct β-actin. Statistical analysis was performed on $2^{-\Delta Ct}$ data and the results were expressed as fold change relative to control [23].

2.5. Fatty acid analysis

Cell lipids were extracted using hexane:isopropanol (3:1) [24] and dried under N2(g). The lipid extracts were then dissolved in chloroform and subjected to thin layer chromatography (TLC) using silica G TLC plates and a mobile phase consisting of hexane: diethyl ether:acetic acid (85:15:1 by vol). To assist in lipid class identification, plates were also spotted with a standard containing phospholipid (PL), cholesterol ester, triacylglycerol (TG) and free fatty acids (Nu-Chek Prep. Inc. Elysian, MN, USA). After development, plates were dried under N2(g), plates were sprayed with 2′,7′-dichlorofluorescein in methanol, and lipids were visualized under UV light. The spots containing TG and PL were scraped into pyrex culture tubes (16 mm × 1.27 mm) with Teflon coated liners, and methylated using 0.5 M sodium methoxide (15 min at 50 °C) with the inclusion of *c*10-17:1 as an internal standard (Nu-Chek Prep). Resulting fatty acid methyl esters were analysed by gas chromatography using a CP-Sil88 column (100 m, 25 µm ID, 0.2 µm film thickness) using a CP-3800 gas chromatograph equipped with a flame ionization detector and a 8400-series autosampler (Varian Inc., Walnut Creek, CA, USA). Hydrogen was used as the carrier gas (1 ml/min initial flow rate, head pressure 25 psi) and the injection port was set at 250 °C, while the detector was set at 260 °C. The temperature program was as follows: initial temperature at 45 °C for 4 min, raised to 175 °C at 13 °C/min and held for 27 min, then to 215 °C at 4.0 °C/min, and finally held at this temperature for 35 min [27]. Individual peaks were identified using reference standards (GLC-603, Nu-Chek Prep. Inc., Elysian, MN, USA) and peak order and retention times reported in the literature [25–27]. The *c*9-16:1/16:0 and *c*9-18:1/18:0 ratios were used to estimate stearoyl-CoA desaturase-1 (SCD1) activity.

2.6. Statistical analysis

Data were analysed using the mixed models procedure of SAS (v 9.3; SAS Institute, Cary, IN, USA) with treatment as the fixed effect and experimental replicate as the random effect. The means were considered to be significantly different at $P < 0.05$ using the Tukey-Kramer multiple comparison test. Data are expressed as means ± SEM.

3. Results

3.1. Cell viability

The viability of HepG2 cells in the presence of 100 µM or 200 µM of fatty acid treatments are shown in Fig. 1. Culturing cells with all 18:1 treatments reduced cell viability over 96 h of incubation compared to BSA control. However, only cells cultured with HT10 or *t*9-18:1 showed significant inhibitory ($P < 0.05$) responses to increased fatty acid concentration (i.e. from 100 µM to 200 µM) occurring from 48 h to 96 h of incubation, and cytotoxic effects of HT10 were more pronounced than *t*9-18:1.

3.2. SFA, cMUFA and PUFA composition and Δ9 desaturation indices

Fatty acid compositions of HepG2 cell TG and PL are presented in Table 3. Treating cells with *t*9-18:1, HT10 or HT11 reduced ($P < 0.05$) SFA (e.g. 16:0 and 18:0) and *c*-MUFA contents comparted to control cells and changes were more pronounced in TG than in PL. Culturing cells with *c*9-18:1 increased its content ($P < 0.05$) mainly at the expense of other *c*-MUFA (e.g. *c*11-18:1 and *c*9-16:1) and SFA, and this effect was more evident in TG than PL. The percentage of n-6 and n-3 PUFA in TG and PL fractions were not affected by any fatty acid treatment. Treatment of cells with *t*9-18:1 or HT10 increased ($P < 0.05$) the *c*9-16:1/16:0 ratio in PL compared to *c*9-18:1 and HT11, but fatty acid treatments had no effect on *c*9-16:1/16:0 ratios in TG. As expected, treatment of cell with *c*9-18:1 led to the greatest *c*9-18:1/18:0 ratios in both TG and PL. The *c*9-18:1/18:0 ratio was increased in PL of cells treated with *t*9-18:1 or HT10 compared to control cells ($P < 0.05$), but was no change was detected with HT11.

3.3. Incorporation of t18:1 isomers and their Δ9 desaturation products

The content of *t*18:1 isomers and their Δ9 desaturation products in TG and PL fractions are presented in Table 4. All fatty acids with a *trans* double bond in cellular lipids originated from exogenous sources (i.e. *t*18:1 treatments) because of their absence from both BSA control and *c*9-18:1 treated cells. All *t*18:1 isomers were incorporated to a much greater extent (1.8–2.5 times) into

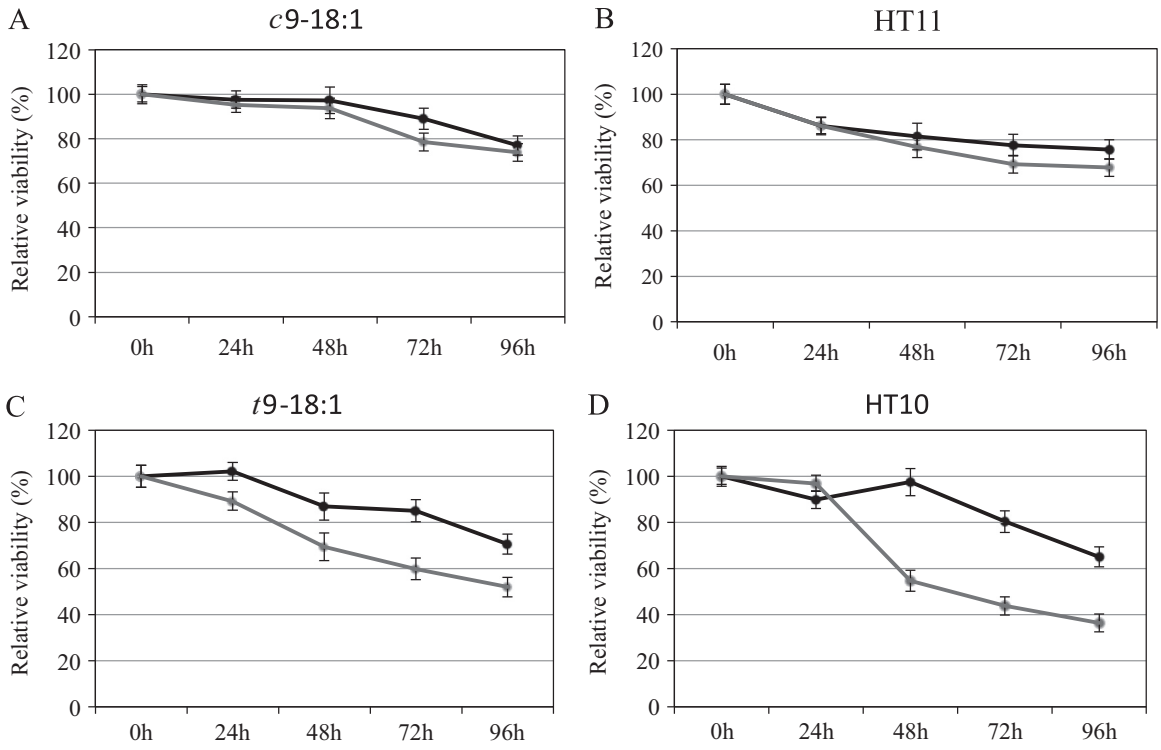

Fig. 1. Viability of HepG2 cells cultured with 100 μM (black) or 200 μM (gray) of *cis*(*c*)9-18:1, *trans*(*t*)9-18:1, HT11 or HT10 during 96 h incubation. Values (mean ± SE; n = 6/treatment) are expressed as percentage changes relative to the BSA control. HT11 = beef *t*-18:1 fraction enriched with *t*11-18:1; HT10 = beef *t*-18:1 fraction enriched with *t*10-18:1.

Table 3
Fatty acid composition (molar %) and Δ9 desaturation ratios in triacylglycerol (TG) and phospholipids (PL) of HepG2 cells cultured with 100 μM of *cis*-9 18:1, *trans*-9 18:1, HT11 and HT10 for 24 h.

Fatty acid[1]	Control	*c*9-18:1	*t*9-18:1	HT11[2]	HT10[2]	SEM[3]
TG						
16:0	30.20^{a}	14.90^{b}	14.21^{b}	18.27^{b}	14.39^{b}	2.00
*c*9-16:1	7.54^{a}	4.44^{b}	5.21^{b}	5.24^{b}	4.03^{b}	0.50
18:0	7.66^{a}	2.76^{b}	2.64^{b}	3.46^{b}	3.50^{b}	0.52
*c*9-18:1	23.53^{b}	61.14^{a}	12.09^{c}	11.85^{c}	10.92^{c}	1.38
*c*11-18:1	21.03^{a}	8.90^{b}	7.75^{b}	8.47^{b}	6.76^{b}	0.61
18:2n6[*]	0.18^{a}	0.21^{a}	0.26^{a}	0.26^{a}	0.27^{a}	0.07
20:4n-6	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
20:5n-3	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
22:6n-3	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
*c*9-16:1/16:0	0.26^{a}	0.30^{a}	0.37^{a}	0.30^{a}	0.28^{a}	0.03
*c*9-18:1/18:0	3.28^{b}	22.40^{a}	4.77^{b}	3.66^{b}	3.16^{b}	0.67
PL						
16:0	22.17^{a}	19.71^{ab}	12.35^{d}	18.24^{bc}	14.85^{cd}	0.91
*c*916:1	12.10^{a}	6.73^{b}	8.03^{b}	7.85^{b}	8.65^{b}	0.56
18:0	8.21^{a}	7.34^{ab}	4.32^{d}	5.28^{cd}	4.93^{bc}	0.36
*c*9-18:1	22.96^{b}	43.36^{a}	15.82^{c}	14.00^{c}	16.69^{c}	0.63
*c*11-18:1	17.35^{a}	9.90^{b}	9.86^{b}	9.41^{b}	9.66^{b}	0.25
18:2n6[*]	1.51^{a}	1.29^{a}	1.55^{a}	1.22^{a}	1.49^{a}	0.10
20:4n-6	3.69^{a}	3.20^{a}	3.65^{a}	3.08^{a}	3.63^{a}	0.18
20:5n-3	0.07^{a}	0.04^{a}	0.07^{a}	0.10^{a}	0.03^{a}	0.04
22:6n-3	2.01^{a}	1.63^{a}	1.74^{a}	1.45^{a}	1.72^{a}	0.14
*c*9-16:1/16:0	0.55^{ab}	0.34^{b}	0.65^{a}	0.43^{b}	0.60^{a}	0.02
*c*9-18:1/18:0	2.89^{c}	5.93^{a}	3.70^{b}	2.66^{c}	3.52^{b}	0.14

[a–d]Means within a row not sharing common letters are significantly different ($P < 0.05$).

[*] 18:2n6 is co-eluting with *c*9,*t*16-18:2.

[1] *c* = *cis*; *t* = *trans*.

[2] HT11 = beef *t*-18:1 fraction enriched with *t*11-18:1; HT10 = beef *t*-18:1 fraction enriched with *t*10-18:1.

[3] Standard error of the mean.

TAG than PL.

Cells treated with HT10 or *t*9-18:1 had 140% more Σ*t*-18:1 in TAG than cells treated with HT11 ($P < 0.05$). In PL, cells treated with *t*9-18:1 had the greatest Σ*t*-18:1 (34%) followed by HT10 (26%) and HT11 (19%). Treatment of cells with HT10 and HT11 led to the incorporation of several minor *t*18:1 isomers due to their presence in treatment fractions, but minor *t*18:1 isomers were not found when cells were treated with (pure) *t*9-18:1.

Several *t*18:1 (e.g. *t*11-, *t*12-, *t*13/*t*14- and *t*15-18:1) in HT10 and HT11 treated cells underwent Δ9 desaturation, and these were detected in both TG and PL. During GC, these were detected as *c*9, *t*11-18:2 and two peaks with co-eluting isomers (*c*9,*t*13-/*c*9,*t*14-18:2 and *c*9,*t*12-/*c*9,*t*15-18:2). In contrast to *t*18:1 isomers, which were preferentially incorporated into TG, the *t*18:1 derived Δ9 desaturation products tended to accumulate in PL. Cells treated with HT11 had the greatest content of *t*18:1 derived Δ9 desaturation products in TG and PL (11.9% and 14.3% respectively), followed by HT10 treated cells (2.0% and 3.6% respectively), while *t*9-18:1 treated cells were devoid of *t*18:1 derived Δ9 desaturation products.

3.4. mRNA expression of key genes involved in fatty acid and cholesterol synthesis

The relative mRNA abundance of key genes involved in *de novo* fatty acid synthesis (ACC, FAS), Δ9 desaturation (SCD1), cholesterol synthesis (HMGCR) and key transcriptional regulators of lipogenesis (SREBP1c) and cholesterol synthesis (SREBP2) are shown in Fig. 2. Culturing cells with *t*9-18:1 or HT10 similarly increased ($P < 0.05$) mRNA expression of FAS, SCD1 and SREBP2 compared to control (BSA) and *c*9-18:1, but culturing with HT11 did not affect expression of these genes. The expression of SREBP1-c was only affected ($P < 0.05$) in *t*9-18:1 treated cells which was increased compared to control (BSA) and other fatty acid treatments.

Table 4
The content (molar %) of *t*-18:1 isomers and their Δ9 desaturation in triacylglycerol (TG) and phospholipid (PL) fractions of HepG2 cells cultured with 100 μM of *trans*-9 18:1, HT11 and HT10 for 24 h.[1]

Fatty acid	t9-18:1	HT11[2]	HT10[2]	SEM[3]
TAG				
Σ*t*-18:1	51.55[a]	35.80[b]	50.65[a]	1.649
*t*6–*t*8-18:1	0.00[b]	1.35[ab]	2.56[a]	0.530
*t*9-18:1	51.55[a]	2.13[bc]	3.38[b]	0.555
*t*10-18:1	0.00[b]	1.88[b]	37.69[a]	1.318
*t*11-18:1	0.00[c]	22.41[a]	3.87[b]	0.695
*t*12-18:1	0.00[c]	3.11[a]	1.12[b]	0.092
*t*13/t14-18:1	0.00[c]	2.17[a]	1.23[b]	0.090
*t*15-18:1	0.00[c]	2.01[a]	0.65[b]	0.082
*t*16-18:1	0.00[b]	0.74[a]	0.16[b]	0.057
Σ*c*9,*t*-18:2	0.00[a]	11.87[b]	2.02[c]	0.695
*c*9,*t*13-/*c*9,*t*14-18:2	0.00[c]	1.28[a]	0.66[b]	0.120
*t*6,*c*9-18:2	0.00[b]	0.23[a]	0.03[b]	0.042
*c*9,*t*15/*c*9,*t*12-18:2	0.00[b]	0.97[a]	0.28[b]	0.077
*c*9,*t*11-18:2	0.00[b]	8.82[a]	1.04[b]	0.711
PL				
Σ *t*-18:1	34.11[a]	19.11[b]	26.68[c]	0.854
*t*6–*t*8-18:1	0.00[a]	0.61[b]	1.55[c]	0.040
*t*9-18:1	34.11[a]	1.64[bc]	2.75[b]	0.454
*t*10-18:1	0.00[b]	0.47[b]	18.21[a]	0.461
*t*11-18:1	0.00[c]	11.63[a]	2.07[b]	0.379
*t*12-18:1	0.00[c]	1.78[a]	0.68[b]	0.064
*t*13/t14-18:1	0.00[c]	1.25[a]	0.78[b]	0.049
*t*15-18:1	0.00[c]	1.21[a]	0.43[b]	0.071
*t*16-18:1	0.00[c]	0.52[a]	0.21[b]	0.031
Σ *c*9,*t*-18:2	0.00[a]	14.30[b]	3.57[c]	0.481
*c*9,*t*13-/*c*9,*t*14-18:2	0.00[c]	1.71[a]	1.08[b]	0.061
*t*6,*c*9-18:2	0.00[b]	0.43[a]	0.10[b]	0.032
*c*9,*t*15/*c*9,*t*12-18:2	0.00[c]	1.21[a]	0.39[b]	0.060
*c*9,*t*11-18:2	0.00[b]	10.36[a]	2.00[c]	0.447

[a-c] Means within a row not sharing common letters are significantly different ($P < 0.05$).

[1] *c*=*cis*; *t* =*trans*; *c*9,*t*-18:2=Δ9 desaturation products of *t*-18:1 isomers.

[2] HT11=beef *t*-18:1 fraction enriched with *t*11-18:1; HT10=beef *t*-18:1 fraction enriched with *t*10-18:1.

[3] Standard error of the mean.

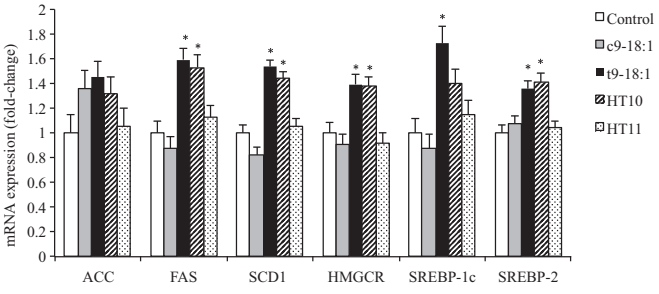

Fig. 2. Effect of culturing HepG2 cells with 100 μM of *cis*(*c*)9-18:1, *trans*(*t*)9-18:1, HT11 or HT10 on relative mRNA expression of genes related to fatty acid (FA) synthesis (ACC: *acetyl*-CoA carboxylase, FAS: fatty acid synthase), Δ9 desaturation (SCD1: Stearoyl-CoA desaturase-1), cholesterol synthesis (HMGCR: 3-Hydroxy-3-Methylglutaryl-CoA reductase), transcriptional regulation of lipogenesis (SREBP1c: sterol regulatory element-binding protein-1c) and cholesterol synthesis (SREBP2: sterol regulatory element-binding protein-2). Values (mean ± SE; n=6/treatment) are expressed as fold changes relative to the control. Within each gene, symbol '*' denotes significant ($P < 0.05$) up-regulation against BSA control (no fatty acid).

4. Discussion

Increasing HT11 concentration from 100 μM to 200 μM did not affect HepG2 cell viability, but viability was reduced in a dose dependent manner by HT10 and *t*9-18:1, and effects were more accentuated in HT10 treated cells. *Trans* 18:1 fatty acids have been shown to induce cell death by both caspase-dependent (e.g. activation of caspase-3, -7 and -9) and caspase-independent pathways (e.g autophagy and intracellular reactive oxygen production) [28–30]. Most previous studies treated cells with *t*9-18:1, but effects of other *t*18:1 isomers on cell viability have not been extensively studied. In primary cardiac myofibroblasts both *t*9-18:1 and *t*11-18:1 reduced cell growth [28], while in HepG2 cells, *t*11-18:1 had less adverse effects than *t*9-18:1 on cell viability [19]. The reason for the greater cytotoxicity of HT10 than *t*9-18:1 in the current study is not known. To our knowledge there is no previous study on the effects of *t*10-18:1 on cell viability, but early studies in rats fed PHVO (i.e. containing a broad spectrum of *t*18:1 isomers) showed that among *t*18:1 isomers, *t*10-18:1 was selectively excluded from PL of tissues, which might be related to its potent cytotoxic properties [31]. Further studies are required to explain the cytotoxic effects of *t*10-18:1.

The reduced content of SFA and *cis*-MUFA in TAG and PL of cells treated with *t*9-18:1, HT10, and HT11 is likely due to their substitution with *t*18:1 isomers [19]. The increased SCD1 index (*c*9-16:1/16:0 ratio) in the PL fraction related to incubation with *t*9-18:1 or HT10 (compared to *c*9-18:1 and HT11) is consistent with up-regulation of SCD1 activity, which is responsible for introducing a double bond between carbon 9 and 10 [32]. Interestingly, culturing with *t*9-18:1 or HT10 increased the *c*9-16:1/16:0 ratio in PL but not in TG. This could be due to the fact that PL are an important component of cell membranes and upregulation of SCD1 (which leads to increased Δ9 desaturation indices) has been suggested to be a defence mechanism used by the cells to restore membrane fluidity when high levels of saturated and *trans* fatty acids are present [33,34]. The lack of increase in SCD1 expression (and SCD1 indices) in cell treated with HT11 could be related to the fact that a significant portion of *t*18:1 isomers in HT11 underwent Δ9 desaturation, which could increase membrane fluidity, and some SCD1 products (e.g. *c*9,*t*11-18:2) are known to provide negative feedback to reduce SCD1 expression [34,35]. The dramatic increase in the *c*9-18:1/18:0 ratio in *c*9-18:1 treated cells was likely because of the addition of exogenous *c*9-18:1 rather than increased Δ9 desaturase activity.

The greater accumulation of *t*18:1 into TG and PL fractions of HepG2 cells treated with HT10 or *t*9-18:1 could also be related in part to the fact that *t*9-18:1 and *t*10-18:1 cannot undergo Δ9 desaturation due to the position of the *trans* double bond [36,37]. In contrast, a significant portions of *t*18:1 in HT11 (e.g. *t*11-, *t*12-, *t*13/14- and *t*15-18:1) underwent Δ9 desaturation giving rise to their desaturation products including *c*9,*t*11-18:2, *c*9,*t*12-/*c*9,*t*15-18:2 and *c*9,*t*13-/*c*9,*t*14-18:2, and these were incorporated to a greater extent in PL than TG.

Consistent with our results, *c*9,*t*11-18:2 was selectively accumulated in liver PL of pigs fed a commercial conjugated linoleic acid mixture [38]. In contrast to 18:2n-6, which is located predominantly at the sn-2 position of PL, conjugated 18:2 isomers that contain one *cis* and one *trans* double bond (e.g. *c*9,*t*11-18:2 and *t*10,*c*12-18:2) are known to be selectively incorporated at the sn-1 position, demonstrating the influence of the *trans* double bond on the positional distribution of its parent molecule [39]. Furthermore, the incorporation of *c*9,*t*11-18:2 at the sn-1 position of PL is suggested to increase membrane fluidity since they mainly replace SFA (e.g. 16:0 and 18:0) which typically occupy the sn-1 position of PL [39]. Thus, Δ9 desaturation of *t*18:1 that can undergo Δ9 desaturation might to some extent alleviate the reduced membrane fluidity caused by their parent molecules. For example, the insertion of a *cis* double bond in *t*11-18:1 by SCD1 reduces the melting point from 44 °C to −4.5 °C [40]. As a consequence, the extent of Δ9 desaturation of *t*18:1 isomers might have important health implications. Interestingly, the content of *t*9-18:1 and *t*10-18:1 (that do not undergo Δ9 desaturation) in the platelets of patients with coronary artery disease have been significantly

correlated with the extent of the disease, while no associations were found with *t*11- or *t*12-18:1 which can undergo Δ9 desaturation [41]. In addition, the anti-carcinogenic and anti-atherogenic properties of *t*11-18:1 have been mainly related to its conversion to *c*9,*t*11-18:2 [42]. However, the potential health effects of Δ9 desaturation products of other *t*-18:1 isomers have not been studied or considered.

Consistent with previous studies using liver cells [18,19], we observed *t*9-18:1 increased the expression of genes involved in fatty acid synthesis (FAS), Δ9 desaturation (SCD1), cholesterol synthesis (HMGCR) as well as key transcriptional regulators of lipogenesis (SREBP1c) and cholesterol synthesis (SREBP2). We are, however, the first to observe gene expression effects of HT10 are similar to *t*9-18:1, except for SREBP1c, which only tended to be upregulated by HT10.

Lipogenic genes including FAS and SCD1 are SREBP1 target genes, while HMGCR, the rate limiting enzyme for cholesterol synthesis, is mainly a target gene of SREBP-2 [43,44]. Recently Shao and Ford [18] reported an increased expression and activity of SREBP-1c in HuH-7 cells cultured with *t*9-18:1. Increased expression of SREBP-1c target genes (ACC, FAS and SCD1) were then also detected, and authors concluded increased hepatic lipogenesis caused by *t*9-18:1 is mainly mediated by an SREBP-1c dependent mechanism. Similarly, the upregulation of HMGCR by *t*9-18:1 could be mediated via SREBP-2. Although we did not measure the SREBP protein or activity in the current study, the greater mRNA expression of SREBP-1c and SREBP2 in cells treated with *t*9-18:1 and HT10 could in part explain the increased expression of lipogenic genes (e.g, FAS and SCD1) and HMGCR. The increased expression of key genes involved in hepatic cholesterol and fatty acid synthesis suggests an increased capacity of liver cells for synthesis of fatty acids and cholesterol needed for VLDL formation (the precursor for LDL). It will now be important to determine whether the in vitro effects of HT10 will parallel the in vivo effects on lipogenesis, lipoprotein metabolism and blood cholesterol (e.g. increased LDL-cholesterol and the LDL-cholesterol/HDL-cholesterol ratio).

In contrast to *t*9-18:1 and HT10, culturing cells with HT11 did not affect the expression of any measured genes compared to control or *c*9-18:1. The muted effect of HT11 on lipogenic gene expression in HepG2 cells could be explained in part by the conversion of *t*11-18:1 (the major isomer in HT11) to *c*9,*t*11-18:2 which has been shown to decrease hepatic SREBP-1c mRNA expression and activity resulting in decreased hepatic lipogenesis in vivo [45]. Consistent with this, feeding *t*11-18:1 has been shown to decrease hepatic lipogenesis and improve blood lipid profiles in animal models of dyslipidemia and metabolic syndrome [46–49], which have been mainly associated with its endogenous conversion to *c*9,*t*11-18:2. Effects of Δ-9 desaturation products of other *t*18:1 isomers, however, remain unknown.

5. Conclusion

The effects of 18:1 isomers on HepG2 cell viability were dependent on double bond position and geometric configuration. All 18:1 isomers resulted in reduced cell viability relative to control. Both *c*9-18:1 and HT11were the least toxic, *t*9-18:1 had dose response increased toxicity, and HT10 had the greatest toxicity. Both *t*9-18:1 and HT10 increased lipogenic gene expression in liver cells compared to *c*9-18:1 and HT11. Overall, results suggest the content and profile of *t*-18:1 in foods should be considered when examining their health effects, and a focus on ruminant-derived *trans* fatty acids should be a priority now that they will become the major source of *trans* fatty acids in the food supply. Additional in vivo studies will be required to determine if present results will translate into recommendations to increase or curtail consumption of meat and dairy products enriched with either *t*10-18:1 or *t*11-18:1.

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Transparency Document. Supplementry material

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